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Prevention of mesangial sclerosis by bone marrow transplantation

J-K Guo[1], TA Ardito[2], M Kashgarian[2] and DS Krause[1]

[1]Department of Laboratory Medicine, Yale School of Medicine, New Haven, Connecticut, USA and [2]Department of Pathology, Yale School of Medicine, New Haven, Connecticut, USA

Previously we have shown that bone marrow (BM) transplantation (BMT) can attenuate progression of and even ameliorate mesangial sclerosis (MS) in *Wt1*-heterozygous mice. However, it is unclear whether BMT performed before the onset of disease will prevent the development of MS. To investigate whether intravenous (i.v.) or intrarenal (i.r.) administration of BM have equal effects on the progression of MS in *Wt1*-heterozygous mice, young *Wt1*-heterozygous mice that had not yet developed renal disease were used as recipients for BMT. After preconditioning with 750 cGy radiation, mice were transplanted with one million wild-type BM via i.v. or i.r. administration. All recipients and untreated controls were assessed for urinary albumin loss, renal pathology, and BM donor-derived renal cells over time. Representative kidney samples were subjected to transmission electron microscopy (TEM) analysis. Interestingly, i.r. and i.v. administration of BM cells gave comparable hematopoietic engraftment levels, and both were able to prevent the onset of MS as assessed by improved lifespan, renal function, renal histology, and TEM analysis. Taken together, we show for the first time that MS can be prevented if BMT is performed before disease onset. Similar therapeutic effects were obtained whether the BM was administered i.v. or i.r.

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Correspondence: *DS Krause, 333 Cedar Street, PO Box 208035, New Haven, Connecticut 06520, USA. E-mail: diane.krause@yale.edu*

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Diffuse mesangial sclerosis (MS) is one of the most common causes of infantile nephrotic syndrome, and is associated with glomerular injury and rapid progression to end-stage renal failure.[1] In mice, decreased levels of Wt1 cause MS, as we and others have observed in *Wt1*+/− mice.[2,3]

Several studies have indicated that bone marrow (BM) contains renoprotective/renotropic cells,[4–8] and we have shown previously that transplantation of wild-type donor BM cells into *Wt1*+/− mice (K-mice, hereafter) attenuates the progression of MS.[9] In this study, we tested whether the route and timing of bone marrow transplantation (BMT) are critical for optimizing the effects of transplanted BM cells.

RESULTS

F1 (FVB/N × C57Bl/6) K-mice serve as a genetic model for late-onset MS

K-mice on a pure FVB/N background develop albuminuria as early as 3 weeks after birth, making them suboptimal for assessing whether BMT can be used to prevent the development of MS in adult mice. To overcome this limitation, we mated FVB/N K-mice with C57Bl/6 wild-type mice to obtain F1 FVB/N × C57Bl/6 K-mice, which have a significantly later onset time and slower progression of MS, comparable to the originally described *Wt1*+/− mice on a mixed background.[2,3] In F1 FVB/N × C57Bl/6 K-mice ($n = 9$) at 2 months after birth, 66.7% were free of albuminuria (the rest with very low level of albuminuria) and all had normal histology. By 6 months, all developed MS with heavy albuminuria (Figure 1).

Intrarenal infusion of BM allows for hematopoietic engraftment

K-mice and their littermates (7–9 weeks old) were divided into four groups: wild-type no-BMT control, K-mice no-BMT control, wild-type BMT recipients, and K-mice BMT recipients (Table 1). One million total wild-type BM cells were transplanted either by tail vein or intrarenal (i.r.) injection into the left kidney. Engraftment was examined by green fluorescent protein (GFP) fluorescence of total nucleated blood cells when mice were killed 6 months post-transplantation. There was no statistical significance between individual groups in terms of BM engraftment levels ($P > 0.05$, Table 1). Next, the level of engraftment of GFP+

podocytes was examined by immunostaining with anti-Wt1 and anti-GFP antibodies on paraffin sections. Again, there was no significant difference in the level of total donor-derived podocytes between different groups ($P>0.05$, Table 1) or donor-derived renal interstitial cells ($P>0.05$, data not shown), indicating that the route of injection did not affect the engraftment of donor cells.

Early BMT can prevent the development of MS

Urinary albumin loss was closely monitored throughout the course of the experiments. Although untransplanted K-mice show a progressive increase in albuminuria, 5 of 10 K-mice (two intravenous, and three i.r.) had no albumin in their urine for up to 6 months post-BMT, and three other recipients had only low levels of albuminuria. For each experimental group at different time points, the average urinary albumin concentration standardized to creatinine levels is shown in Figure 1. Of these eight K-mice that had little to no albuminuria, seven had normal histology (Figure 2a). The three other transplanted K-mice with higher levels of albuminuria had slight pathology with a small increase in extracellular matrix in the glomerular tuft, but otherwise normal tubular structure and interstitium. These 10 K-mice recipients had an average histology score of 0.225. In contrast, the untransplanted K-mice all developed severe MS (mean histology score of 3.5) within the same 6-month period. The results of an intensive podocyte number count on Wt1-immunostained slides confirmed that BMT can prevent the loss of podocytes (Figure 2b). Immunofluorescent study of the diaphragm slit using anti-nephrin antibody also revealed the beneficial effect of BMT on podocyte morphology in K-mice (data not shown), as the staining pattern of nephrin is a sensitive indicator of podocyte status. Moreover, TEM analysis indicated that BMT using wild-type donors helped to maintain normal podocyte foot processes in the *Wt1*+/− recipients. There was no evidence of adverse effects of BMT on glomerular mesangial cells, glomerular basement membrane, or endothelial cells (Figure 2c). More important, the two kidneys of individual i.r. recipients showed identical histology even though only one kidney had BMT administered, suggesting that the beneficial effect was derived from circulating BM cells. The left and right kidneys had identical levels of donor-derived cells as well (Figure 3).

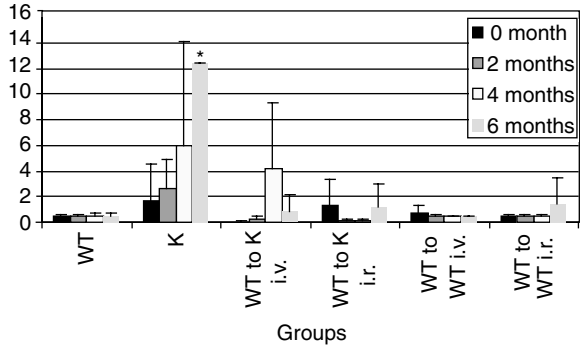

Figure 1 | Quantification of urinary albumin by enzyme-linked immunosorbent assay. *X* axis: six experimental groups, *y* axis: albumin level standardized to creatinine. WT**:** wild-type untreated group showing baseline levels of albuminuria. K: untreated K-mice showing significant urinary albumin loss. WT to K i.v.: K-mice recipients with wild-type BM via i.v. WT to K i.r., K-mice recipients with wild-type BM via i.r. WT to WT i.v.: wild-type recipients with wild-type BM via i.v. WT to WT i.r.: wild-type recipients with wild-type BM via i.r. Samples collected 0, 2, 4, and 6 months post-BMT were assayed. *No s.e.m. available because the number of mice for this time point dropped to two.

DISCUSSION

The finding that 70% of K-mice recipients ($n=10$) had completely normal histology when BMT was performed before disease onset suggests that it is possible to prevent, at least for 6 months, the development of MS in mice heterozygous for the *Wt1* locus. Moreover, we found that there is no advantage to delivering BM cells directly into the kidney.

These data provide further evidence that BMT can attenuate and even prevent the development of MS owing to Wt1 insufficiency, regardless of the route of transplantation. The finding that wild-type and K recipients have comparable low levels of donor-derived podocyte engraftment as observed here and in our previous studies[9] indicates that pre-existing severe renal injury does not enhance donor-derived cells to become podocytes and the therapeutic/preventive effect may take place independent of donor-derived renal epithelial cells.

Table 1 | Summary of BMT results

Groups	*N*	Route of BM injection	Engraftment in BM	Survival (6 months post-BMT) (%)	Donor-derived podocytes[a] (%)
WT	3	N/A	N/A	100	N/A
K	3	N/A	N/A	67.8	N/A
BMT/WT	6	i.r. (*n*=2)	65±7.1%	100	0.15±0.07
		i.v. (*n*=4)	80.3±6.5%	100	0.86±0.56
BMT/K	10	i.r. (*n*=5)	81.3±8.5%	100	0.50±0.16
		i.v. (*n*=5)	86.3±4.2%	100	0.81±0.86

BM, bone marrow; BMT, bone marrow transplant; BMT/WT, wild-type recipients; BMT/K, K-mice as recipients; N/A, not applicable; i.r., intrarenal.
All BM was from wild-type donors.
[a]Percentage of podocytes (Wt1+ nuclei within the glomeruli) that were GFP$^+$.

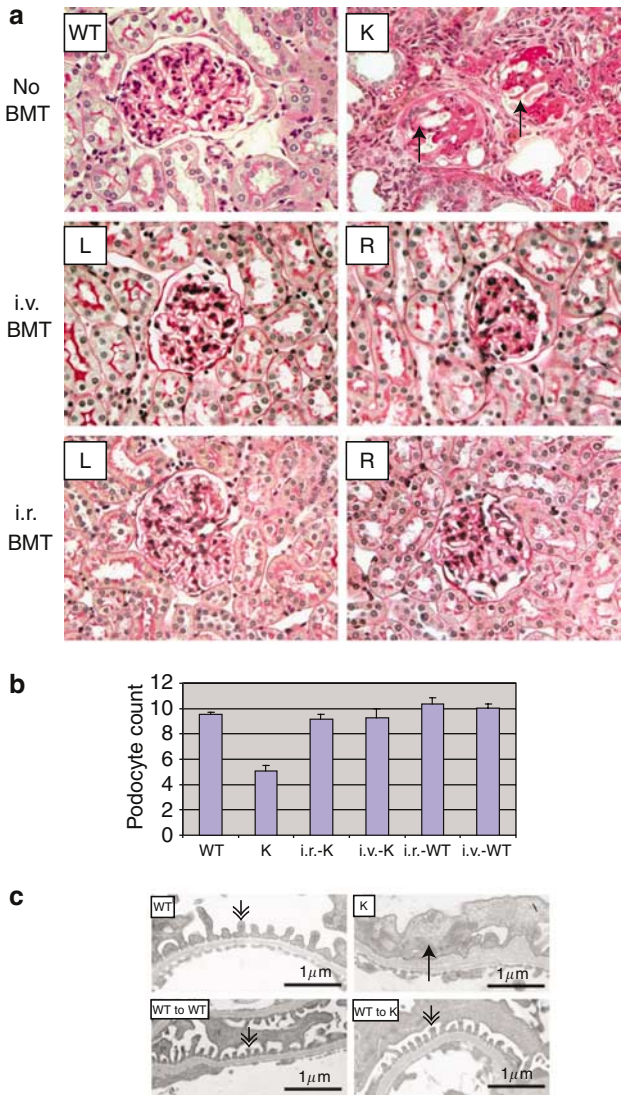

Figure 2 | BMT prevents the development of MS. (**a**) Comparison of kidney histology of four different groups 6 months post-BMT. Upper row: WT showing normal histology; K: untreated, age-matched *Wt1* + /− mouse showing severe sclerosis (arrow) of glomerular tuft, tubular dilation with protein casts, and interstitial inflammation; middle row: a representative K-recipient of BMT via i.v. showing left (L) and right (R) kidneys that have normal histology; bottom row: a representative K-recipient of BMT via i.r., showing left (L) and right (R) kidneys that are both normal, although the BM was injected into left kidney. Paraffin sections (3 μm), original magnification $\times$ 200. (**b**) Podocyte number. K-mice have about half the number of podocytes per cross-section of glomerulus compared to wild-type controls. The BMT recipients via i.v. or i.r. all show normal podocyte numbers 6 months after BMT. (**c**) TEM analysis of glomeruli. Untreated K-mice have wide-spread focal effacement of podocyte foot processes (arrow) and thickening of the glomerular basement membrane. In comparison, BMT recipients (either wild-type or K-mice) maintained (double arrow) normal podocyte foot processes, glomerular basement membrane, and endothelial fenestration; thus, the integrity of the ultrafiltration barrier is preserved. Bar = 1 μm.

Further studies will help determine the underlying mechanism of this beneficial effect, especially whether donor-derived renal interstitial cells account for the therapeutic effects of BMT.

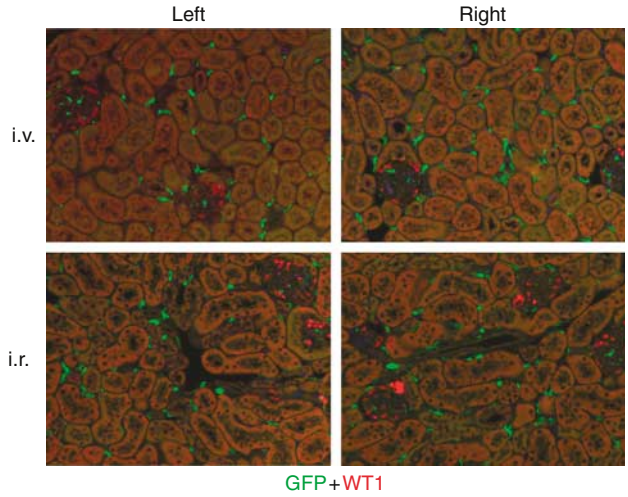

Figure 3 | i.r. and i.v. transplantation of BM leads to comparable levels of renal engraftment of donor GFP$^+$ cells. Upper row: K-recipient of i.v. BMT; lower row: K-recipient of i.r. BMT. Green: GFP staining and red: Wt1 staining. Left and right kidneys show similar staining pattern in either group (i.v. or i.r.). Paraffin section (3 μm), original magnification $\times$ 200.

MATERIALS AND METHODS

Mouse care and BMT

Wt1 + /− mice (K-mice) were genotyped as described.[2] In this study, we used F1 K-mice (FVB/N $\times$ C57Bl/6) owing to the late onset of MS on this mixed genetic background.[2,3] GFP mice (C57BL/6-Tg(ACTbEGFP)1Osb/J) were from Jackson laboratory (Bar Harbor, Maine)[10] F1 GFP mice (C57BL/6-Tg(ACTbEGFP)1Osb/J $\times$ FVB/N) were used as donors to match the genetic background of the recipients. All mouse protocols were approved by the Yale University Institutional Animal Care and Use Committee. Donor BM isolation, and BMT were carried out as described.[6,11] Two-month-old mice were sublethally irradiated with 750 cGy. For i.r. injection, an abdominal incision was made to expose the left kidney. Using a 1 ml syringe and 30 G needle, 50 μl (2×10^7 cells/ml) BM cells was injected into the renal parenchyma about 2–3 mm beneath the renal capsule. The needle was kept in position for 15 s before withdrawal to prevent leakage of injected cells and warm 1 $\times$ phosphate-buffered saline-dampened non-woven cloth was gently pressed down for 1 min to stop potential bleeding. Other recipients received an equal number of BM cells (5×10^6 cells/ml, 200 μl) via tail vein injection. Engraftment was verified at the time of being killed by fluorescence-activated cell sorter analysis of peripheral blood. GFP$^+$ donors are at times referred to as wild-type in terms of the *Wt1* locus in this study.

Urine analysis

Urine albumin and creatinine levels were quantified as described previously.[9]

Histology, TEM, fluorescent immunostaining, and Y-fluorescent *in situ* hybridization

After killing, kidneys were fixed in buffered formalin for 12 h, embedded in paraffin, sectioned at 3 μm and periodic acid-Schiff stained. To compare the degree of renal pathology, we used a semiquantitative system on a 0–5 scale with the best (score = 0) being normal histology, and the worst score of 5 being completely

sclerotic kidney as previously described.[9] For TEM analysis, mice were perfused with 2% glutaraldehyde and 2% paraformaldehyde. Kidneys were processed for TEM according to standard protocol. Sections were viewed on a Zeiss EM-910 (Carl Zeiss Inc., Oberkochen, Germany) electron microscope at 80 kV.

Although GFP was the primary tag for tracking the donor-derived cells, fluorescent *in situ* hybridization for the Y chromosome was also performed as described previously[6] to verify the results when female recipients of male BM cells were used.

Immunostaining on paraffin-embedded tissue was performed as described,[9,12] using rabbit polyclonal antibody against Wt1 (Santa Cruz Biotechnology, CA, USA), rabbit polyclonal anti-nephrin antibody (a kind gift from Larry Holzman, Ann Arbor, MI, USA), goat anti-GFP (Abcam, Cambridge, MA, USA) and rat anti-mouse CD45 (BD Biosciences, NJ, USA).

Statistical analysis

Data were analyzed using Student's *t*-test with $P<0.05$ regarded as statistically significant.

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